Hepatitis delta virus ribonucleoproteins shuttle between the nucleus and the cytoplasm

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ABSTRACT

Hepatitis delta virus (HDV) infection of individuals infected with hepatitis B virus (HBV) is associated with more severe liver damage and an increased risk of fulminant disease. HDV is a single-stranded RNA virus that encodes a single protein, the delta antigen, which is expressed in two forms, small (S-HDAg) and large (L-HDAg). Here we show that although HDV ribonucleoproteins are mainly detected in the nucleus, they are also present in the cytoplasm of cells infected with HDV or transfected with HDV cDNA. Making use of an heterokaryon assay, we demonstrate that HDV ribonucleoproteins shuttle continuously between the nucleus and the cytoplasm. In the absence of HDV RNA, both forms of the delta antigen are retained in the nucleus, whereas in the absence of the delta antigen, HDV RNA is predominantly detected in the cytoplasm. Coexpression of HDV RNA and S-HDAg (which binds to the viral RNA and contains a nuclear localization signal) results in nuclear accumulation of the viral RNA. This suggests that HDV RNA mediates export of viral particles to the cytoplasm whereas the delta antigen triggers their reimport into the nucleus.

Keywords: HBV; HDV; nucleocytoplasmic transport; virus

INTRODUCTION

The hepatitis delta virus (HDV) was first discovered in 1977 during study of liver biopsies of patients infected with hepatitis B virus (HBV; Taylor, 1992; Monjardino & Lai, 1993). It was further demonstrated that HDV infection was associated with increased severity of HBV-associated liver disease (Govindarajan et al., 1984). The biological association between the two viruses is due to the fact that the outer coat of HDV consists of HBV surface proteins, which explains the required presence of HBV for HDV productive infection and transmission (Smedile et al., 1994). HDV has a single-stranded RNA genome of about 1700 nt, which is about 70% self-complementary, thus forming a highly base-paired rodlike structure (Taylor, 1992). HDV RNA encodes two protein forms of the hepatitis delta antigen (HDAg), the small (S-HDAg or δ Ag-S) and large (L-HDAg or δ Ag-L). The large form is produced by an editing mechanism, which changes the UAG stop codon of the small antigen to a UGG tryptophan codon leading to the readout of 19 additional amino acids

(Taylor, 1992). The template for RNA editing is the anti-genomic RNA molecule and the enzyme involved is thought to be a cellular double-stranded RNA-specific adenosine deaminase (dsRAD/DRADA/ADAR1; Casey & Gerin, 1995). The two forms of the delta antigen have very distinct functions regarding the biology of HDV. S-HDAg is a protein of 195 amino acids and a molecular weight of 24 kDa, and is essential for HDV replication, whereas L-HDAg contains 214 amino acids and a molecular weight of 27 kDa, represses HDV replication (Chao et al., 1990), and is required for packaging viral particles (Ryu et al., 1992). Both forms of delta antigen have nuclear localizing sequence (NLS) and RNA-binding domains (Monjardino & Lai, 1993). Furthermore, the additional 19 amino acids of the L-HDAg are necessary for the interaction with the HBV surface proteins (Lee et al., 1995).

Upon entering into the cell, and after being uncoated, the HDV RNP complex is transported to the nucleus, a process mediated by the delta antigen (Chou et al., 1998). Once in the nucleus, the HDV RNPs initiate replication by an RNA transcription mechanism, which is thought to involve the host cell RNA polymerase II (Filipovska & Konarska, 2000). The current model of HDV replication states that the incoming HDV genomes are templates for rolling circle replication result-

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ing in the production of multimeric antigenomes. These nascent multimeric molecules harbor ribozymes capable of self-cleaving segments of monomer length. An antigenomic linear monomer released by two self-cleavage events is then ligated to generate a circular molecule, which then becomes the template for synthesis of genomic circular molecules by a similar rolling circle mechanism (Taylor, 1992).

Although the interaction between HDV and HBV is crucial for pathogenesis, very little is known about how it occurs in the infected cell. Several studies have examined the intracellular distribution of the HDV antigen and RNA and there is general consensus that viral ribonucleoproteins (RNPs) are mainly localized in the nucleus (Cunha et al., 1998; Bell et al., 2000). In contrast, the HBV surface antigens are exclusively detected in the cytoplasm (Eble et al., 1986). Given this spatial separation, the main goal of our work was to investigate how these two components come together in order to form complete HDV particles capable of propagating infection. Making use of heterokaryon assays, we show that HDV RNPs are continuously shuttling between the nucleus and the cytoplasm. We propose a model for nucleocytoplasmic trafficking of HDV virion particles that relies on the presence of an export signal in the viral RNA and an import signal in the delta antigen.

RESULTS

Intracellular distribution of HDV

We started by analyzing the intracellular distribution of delta proteins and HB surface antigen in liver biopsies

from patients infected with both hepatitis B and delta viruses. The histological samples were derived from patients with liver transplants for terminal HDV cirrhosis, as previously described (David et al., 1993). As depicted in Figure 1A, a subset of hepatocytes are simultaneously labeled by antibodies directed against delta proteins (red staining) and HB surface antigen (dark purple staining). In these cells, the delta polypeptides are localized in the nucleus, whereas the HBs antigen is detected in the cytoplasm. In addition, we observe cells that are exclusively immunoreactive for either delta or HBs antigens (Fig. 1B). Unexpectedly, some cells expressing only delta antigens are stained both in the nucleus and cytoplasm (Fig. 1C–E), suggesting that S-HDAg and L-HDAg can localize to the cytoplasm in the absence of HBsAg expression. Consistent with these results, localization of delta antigen to the cytoplasm of infected mouse hepatocytes has been recently reported (Chang et al., 2001).

To further investigate if HDV ribonucleoprotein particles (RNPs) are exported from the nucleus to the cytoplasm, we analyzed Huh7-D12 cells. These cells are derived from the human hepatoma cell line Huh7, which was stably transfected with the plasmid pSVL(D3) that bears a trimer of full-length genomic HDV cDNA cloned in the eukaryotic expression vector pSVL (Cunha et al., 1998). This cDNA was originally synthesized from total liver RNA derived from an infected woodchuck inoculated with an HDV-containing human serum (Kuo et al., 1989). Immunofluorescence of Huh7-D12 cells using antibody B3 shows predominant labeling of intranuclear foci (Fig. 2A,B). To detect the viral RNA, *in situ* hybridization was performed using pSVL(D3) as a probe. As previously described (Cunha et al., 1998), pSVL(D3)

FIGURE 1. Nucleo-cytoplasmic localization of HDV and HBV antigens in infected human liver. Paraffin sections were obtained from the liver of patients transplanted for terminal HDV cirrhosis with recurrent HDV and HBV infection (David et al., 1993). The sections were double-labeled for the delta (red color) and HBs antigens (dark purple color). **A:** A group of hepatocytes expressing simultaneously delta and HBs antigens. **B,C:** Islets of cells expressing only HBs antigens, surrounded by dispersed cells expressing exclusively delta antigens. **D,E:** Details of two cells expressing exclusively delta antigens, which appear localized both in the nucleus and in the cytoplasm.

FIGURE 2. Subcellular distribution of HDV antigens and RNA. Huh7-D12 cells were double-labeled with B3 antibody directed against the small and large forms of the delta antigen (**A** and **B**, red staining), and plasmid pSVL(D3), which hybridizes to both genomic and antigenomic viral RNA (**C**, green staining). The delta proteins are predominantly localized in the nucleus. In some cells, the labeling is concentrated in nucleoplasmic foci, whereas in others there is additional staining of the nucleolus. Hybridization with pSVL(D3) reveals the HDV cDNA integrated in the host chromatin (**C** and **D**, arrows; see Cunha et al., 1998), as well as brightly labeled nucleoplasmic foci. Superimposition of red and green images (**C**) reveals a yellowish color, indicating that the delta antigen and RNA colocalize in the same nucleoplasmic foci. **E–H**: A series of optical sections along the z axis of Huh7-D12 cells double-labeled for delta antigens (red staining) and RNA (green staining). Arrows in **E** point to sites of HDV cDNA integration (green staining). Arrowheads in **G** and **H** indicate colocalization of viral proteins and RNA in the cytoplasm (yellowish staining). Bar: 10 μ m.

hybridizes with both the viral RNA and with the viral cDNA integrated in the genome of the Huh7-D12 cells (Fig. 2C,D,E, green dots indicated by arrows). Simultaneous detection of HDV antigen and RNA shows colocalization of these two components in nucleoplasmic foci, and in the cytoplasm (Fig. 2E–H, arrowheads). The finding that both HDV antigens and RNA are localized to the cytoplasm strongly suggests that viral RNPs are exported from the nucleus to the cytoplasm in the absence of HBV surface antigens.

HDV RNPs shuttle continuously between the nucleus and the cytoplasm

To further study the nucleocytoplasmic transport of HDV RNPs, we made use of an interspecies heterokaryon assay (Pinol-Roma & Dreyfuss, 1992). The migration of the HDV particles was monitored in human–mouse heterokaryons produced by polyethylene glycol-induced fusion of Huh7-D12 and NIH 3T3 cells (Fig. 3A–D). These heterokaryons were kept in culture for 5 h in the presence of the protein synthesis inhibitor emetine in order to exclude import of proteins newly synthesized in the cytoplasm of the heterokaryon into the murine nucleus. The human nuclei are easily distinguished from murine nuclei based on different heterochromatin pat-

terns revealed by DAPI staining (Fig. 3A, arrowhead; see also Fig. 5E,G, arrows). To identify HDV RNPs, cells were double-labeled for the viral antigen (Fig. 3B) and viral RNA (Fig. 3C). As depicted in Figure 3B–D, both the delta antigen and RNA are detected in the murine nuclei. This suggests that following membrane fusion the HDV RNPs migrated from the Huh7-D12 nuclei to the cytoplasm of the heterokaryons and from there they were imported to the NIH 3T3 nuclei. Interestingly, the HDV RNPs present in the murine nuclei tend to concentrate in foci similar to those observed in Huh7-D12 cells.

In conclusion, these data show that the HDV RNPs shuttle between nucleus and cytoplasm, and that both import to the nucleus and export to the cytoplasm are independent of the presence of HBV surface antigens.

The delta antigens are retained in the nucleus when expressed in the absence of viral genomic RNA

Having established that HDV RNPs are exported to the cytoplasm, we next asked whether S-HDAg or L-HDAg harbors a nuclear export signal (NES). Huh7 cells were transiently transfected with plasmids encoding either

FIGURE 3. HDV RNPs shuttle between nucleus and cytoplasm. Huh7-D12 cells were fused to mouse 3T3 fibroblasts to form interspecies heterokaryons. DAPI staining of DNA allows to distinguish human from murine nuclei, because the nuclei from mouse cells contain prominent clumps of heterochromatin (**A**). Cells were double-labeled for delta antigen (**B**, red staining) and RNA (**C**, green staining). **D**: A superimposition of red and green images. Arrowheads indicate the Huh7-D12 cell nucleus in this heterokaryon. Arrows point to sites of HDV cDNA integration. Bar: 10 μ m.

δ Ag-S or δ Ag-L in the absence of genomic HDV RNA (Cunha et al., 1998; Fig. 4C). Given that nuclear pore complexes allow the diffusion of molecules smaller than 60–70 kDa (reviewed in Gorlich & Kutay, 1999), we first investigated whether the delta polypeptides leak to the cytoplasm by passive diffusion. To address this question, a temperature-shift experiment was performed as described (Michael et al., 1995). The rationale for this

assay is that at 4 °C, both receptor-mediated nuclear import and export are blocked, whereas passive diffusion continues to occur. As a positive control, we used GFP fused to a classical NLS (the SV40 large T antigen NLS). GFP is a small protein (approximately 27 kDa) that can freely diffuse through the nuclear pores. However, due to the presence of the NLS, the protein is actively imported by an energy-dependent carrier-

FIGURE 4. Delta antigens do not leak out to the cytoplasm by passive diffusion. Huh7 cells were transfected with GFP-NLS, treated with a protein synthesis inhibitor (40 μ g/mL emetine) for 3 h and then incubated in medium containing emetine for another 3 h at 37 °C (**A**) or 4 °C (**B**). Total proteins from Huh7 cells transfected with pSVLAg-S (lane 1), Huh7 cells transfected with pSVLAg-L (lane 2), Huh7-D12 cells (lane 3), and nontransfected Huh7 cells (lane 4) were separated in a 12% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and incubated with the B3 antibody (**C**). Huh7 cells transfected with either pSVLAg-L (**D**, **E**) or pSVLAg-S (**F**, **G**) were treated with emetine and incubated at 37 °C or 4 °C, as indicated. Bar: 10 μ m.

mediated mechanism. At physiological temperature, GFP-NLS diffuses out of the nucleus and is rapidly reimported. As a result, the protein is exclusively detected in the nucleus (Fig. 4A). In contrast, at low temperature, the GFP-NLS fusion protein continues to leak out of the nucleus, but because the NLS-mediated import is impaired, the protein progressively accumulates in the cytoplasm (Fig. 4B). In striking contrast to the results obtained with GFP-NLS, both the small and large forms of the delta antigen are exclusively localized in the nucleus of Huh7 cells incubated at low temperature (Fig. 4D–G).

Having ruled out the possibility that delta proteins leak out to the cytoplasm by passive diffusion through the nuclear pores, we next asked whether S-HDAg or L-HDAg shuttle between nucleus and cytoplasm by an active mechanism. Huh7 cells were transfected with either pSVLAg-S or pSVLAg-L and fused with NIH 3T3 mouse cells. The resulting heterokaryons were kept in culture for 3 to 5 h in the presence or absence of emetine (Fig. 5). When protein synthesis was inhibited, neither S-HDAg (δ Ag-S) nor L-HDAg (δ Ag-L) was detected in the murine nuclei of heterokaryons (Fig. 5A,C,E–H). Double-labeling with a monoclonal antibody specific for human hnRNP C, a protein that does

not shuttle (Pinol-Roma & Dreyfuss, 1992), produces similar results, that is, exclusive staining of the human nucleus (Fig. 5B,D). In the absence of emetine, δ Ag-S, δ Ag-L, and hnRNP C are readily detected in the murine nuclei, indicating that when these proteins are synthesized in the cytoplasm of the heterokaryons, they can be imported into either the human or the mouse nucleus (Fig. 5I–L). In conclusion, these results show that both the small and large forms of the delta antigen fail to be exported from the nucleus in the absence of HDV genomes. One possibility is that binding of delta proteins to the RNA is required to expose an export signal on the antigen. Alternatively, the viral RNA contains the export signal.

HDV genomic RNA is exported to the cytoplasm in the absence of the delta antigen

To test whether the signal required to mediate export of HDV particles to the cytoplasm is present in the viral genomic RNA, we made use of plasmid pDL542, which contains a 2-nt deletion at position 1434 to 1435 and does not support production of delta antigen (Lazinski & Taylor, 1994). Expression of pDL542 gives rise to

FIGURE 5. Delta antigens are retained in the nucleus when expressed in the absence of HDV RNA. Huh7 cells transfected with either pSVLAg-L or pSVLAg-S were fused to mouse 3T3 fibroblasts to form heterokaryons. Huh7 cells were treated with emetine for 3 h before fusion. After fusion, the cells were kept in culture for 5 h in the presence of emetine (A–H). Alternatively, the cells were incubated in the absence of emetine (I–L). Cells were simultaneously labeled with rabbit serum B3 (A, C, I, K), monoclonal antibody 4F4 directed against the nonshuttling hnRNP C protein (B, D, J, L), and DAPI (E, G). F and H depict phase contrast images. Arrows in E and G point to clumps of heterochromatin characteristically observed in murine nuclei. Arrowheads in I and K indicate a Huh7 cell nucleus. Bar: 10 μ m.

small 1.2 kb RNA circles that are unstable and do not undergo replication. Huh7 cells were transiently transfected with pDL542 and in situ hybridization was performed using pSVL(D3) as a probe. As depicted in Figure 6A, viral RNA is predominantly detected in the cytoplasm of transfected cells. A similar distribution is observed when Huh7 cells are transfected with pDL481 (Fig. 6B) that expresses full-length antigenome RNA (Lazinski & Taylor, 1994). Surprisingly, however, both full-length genomic and antigenomic HDV RNA expressed in HeLa cells is exclusively detected in the nucleus (Fig. 6C and data not shown).

After cotransfection of Huh7 cells with both pDL542 and a plasmid encoding the δ Ag-S protein, which binds to the 1.2-kb genomic RNA (Lazinski & Taylor, 1994), a significant fraction of the genomic RNA is detected in the nucleus. Double-labeling experiments show that the viral RNA is distributed throughout the nucleoplasm excluding the nucleolus (Fig. 6D,F), whereas the small form of the delta protein localizes throughout the nucleoplasm with additional concentration in the nucleolus (Fig. 6E,F).

Taken together, the data suggest that the HDV RNA interacts with the cellular machinery responsible for

nucleo-cytoplasmic export, independently of the delta antigen. Association of the viral RNA with the small form of the delta antigen, which contains an NLS, most likely triggers import of the RNP into the nucleus. The different results observed in hepatoma and HeLa cells further suggest that the cellular transport factors involved in the nuclear export of HDV RNA are cell-type specific.

DISCUSSION

In the present work, we show that, although at steady state HDV ribonucleoprotein particles accumulate predominantly in the nucleus, they continuously shuttle between nucleus and cytoplasm. The data further suggest that HDV genomic RNA mediates export of the viral particles to the cytoplasm, whereas import of the RNPs into the nucleus is probably triggered by the NLS located in the delta antigen.

We have previously shown that when either the small or the large forms of the delta antigen are transiently expressed in Huh7 cells, both proteins are exclusively localized to the nucleus (Cunha et al., 1998). However, given their small size, these proteins could, in principle,

FIGURE 6. HDV RNA is exported to the cytoplasm in the absence of delta antigens. Huh7 (A, B) and HeLa cells (C) were transfected with pDL542 (A) or pDL481 plasmids (B, C) that express full-length genomic and antigenomic HDV RNA, respectively. Alternatively, Huh7 cells were cotransfected with pDL542 and pSVLAg-S (D–F). Viral RNA was detected by in situ hybridization using pSVL(D3) as a probe (A, B, C, D, E, F, green staining), whereas the delta antigen was visualized with B3 antibody (E, F, red staining). F depicts a superimposition of green and red images. Bar: 10 μ m.

leak out of the nucleus by passive diffusion through the nuclear pores. In fact, nuclear pore complexes form aqueous channels with a diameter of approximately 9 nm that allow diffusion of small molecules. Nevertheless, even proteins or RNAs that are smaller than 20–30 kDa normally cross the nuclear pore complex using an active, carrier-mediated mechanism that consumes energy (reviewed in Gorlich & Kutay, 1999). The data presented here show that neither S-HDAg nor L-HDAg expressed in the absence of HDV genomes exits the nucleus by passive diffusion. Moreover, at physiological temperature, neither of these proteins is exported from the nucleus. This suggests that delta proteins bind to cellular components within the hepatic cell nucleus, thus preventing diffusion to the cytoplasm. Intriguingly, Chang and coworkers have recently reported nucleocytoplasmic shuttling of L-HDAg transiently expressed in HeLa cells, and identified a subdomain in the protein that can function as a nuclear export signal in *Xenopus* oocytes (Lee et al., 2001). This apparent discrepancy may be due to cell-type specificities. In fact, we have additionally observed that HDV RNA is either exported to the cytoplasm or retained in the nucleus depending on whether it is expressed in hepatoma or HeLa cells (Fig. 6B,C).

Although both forms of the delta antigen are retained in the nucleus of Huh7 cells devoid of HDV genomes, these proteins shuttle continuously between the nucleus and cytoplasm of Huh7-D12 cells. Thus, expression of the HDV genome overcomes the nuclear retention of the delta antigen, raising the possibility that viral RNA bears the signal required for nuclear export of the viral RNPs. Consistent with this view, expression of a plasmid encoding a mutated form of the HDV genome (pDL542) that does not support synthesis of the delta antigen (Lazinski & Taylor, 1994) produces RNAs exclusively localized to the cytoplasm (Fig. 6A). As synthesis of HDV RNA takes place in the cell nucleus (Monjardino & Lai, 1993), this implies that the viral RNA is exported from the nucleus to the cytoplasm in the absence of the delta antigen. Coexpression of δ Ag-S, which contains an NLS and binds to the mutant RNA (Lazinski & Taylor, 1994), leads to accumulation of the RNA in the nucleus (Fig. 6D–F), suggesting that delta proteins trigger the import of viral ribonucleoproteins back into the nucleus. This would explain why in cells infected with HDV in the absence of HBsAg (Fig. 1B) or transfected with HDV cDNA (Fig. 2A–D), both the viral antigen and RNA are predominantly localized to the nucleus.

According to the current model for HDV pathogenesis, virus spread and reinfection can only occur after replicating genomes (coding for the small form of delta antigen) are edited, leading to the generation of L-HDAg. Once the large form of the delta antigen is produced, further RNA replication is inhibited. As viral particles are released, the intranuclear pool of L-HDAg may de-

crease more rapidly than S-HDAg, thus allowing replication to resume (Monjardino & Lai, 1993). Although δ Ag-L plays an essential role in packaging HDV particles, virus assembly requires in addition the surface proteins from HBV. The surface antigens from HBV (HBsAg) include small (S), middle (M), and large (L) polypeptides, all of which are components of the virus envelope. The nascent HBsAg S polypeptides are rapidly inserted into the endoplasmic reticulum bilayer as transmembrane proteins. Following insertion, the transmembrane monomers aggregate and host proteins are excluded from the complex. The aggregate is then transposed into the lumen by a process of extrusion or budding and the resulting viral particles are rapidly secreted from the cell using the normal pathways of vesicular transport (reviewed in Ganem, 1991). Expression of the S protein alone is sufficient for HDV virion assembly (Sureau et al., 1992), but the HBsAg L protein is required for infectivity of HDV virions (Sureau et al., 1992). Based on the data presented here, we propose that HDV virion particles exit the nucleus due to the presence of a signal on the viral RNA that is recognized by the cellular export machinery (Fig. 7). However, because both forms of the delta antigen present in the viral RNPs contain an NLS, the complexes are rapidly reimported into the nucleus. This explains why at steady state, the viral RNPs are predominantly localized to the nucleus. On the other hand, if the cell contains aggregates of HBV surface proteins inserted in the endoplasmic reticulum membrane, some HDV virion particles will bind to these complexes, assemble a virus envelope, and enter the cellular secretory pathway in order to propagate the infection. It is also conceivable that newly synthesized delta antigens may directly assemble with the HBV surface proteins at the endoplasmic reticulum before ever entering the nucleus and binding to HDV RNA. Consistent with this prediction, expression of HDAg-S, HDAg-L, and HBsAg S results in assembly of viruslike particles, which contain no HDV RNA (Ryu et al., 1992).

In summary, our results suggest that HDV RNA contains a putative element that interacts with cellular nuclear export factors. Viruses are known to have evolved mechanisms for export of their RNAs, which are distinct from those used to export cellular RNAs. In the particular case of retroviruses, it is well established that transport mechanisms rely on the presence of an RNA element in the viral genome, which binds either viral or cellular factors that promote nuclear export (reviewed in Izaurralde & Adam, 1998). For example, the simian retrovirus type 1 RNA contains a so-called constitutive transport element (CTE), which folds into an extended RNA stem-loop structure (Ernst et al., 1997) that binds to the cellular export factor TAP (Braun et al., 1999). Whether an equivalent transport element is present in HDV RNA remains to be determined. Unraveling the mechanisms involved in export of HDV from the nu-

FIGURE 7. Proposed model for nucleocytoplasmic traffic of HDV particles. The HDV genomic RNA is replicated and transcribed in the nucleus. The single 700-nt mRNA transcribed from the viral genome is edited and exits the nucleus to produce the small and large forms of the delta antigen. Due to the presence of an NLS, these proteins are rapidly imported into the nucleus. Within the nucleus, both forms of the antigen bind to HDV RNA. The small form participates in viral RNA replication, whereas the large form suppresses replication and promotes the assembly of virion particles. These particles may then exit the nucleus due to the presence of a putative signal on the viral RNA that is recognized by cellular export factors (exportins). In the cytoplasm, the NLS present in the delta antigen is recognized by import receptors (importins). Consequently, the viral RNP particle is reimported into the nucleus. However, if the cell contains HBV surface proteins at the endoplasmic reticulum, some HDV virion particles will bind to these complexes, assemble a virus envelope, and enter the cellular secretory pathway in order to propagate the infection. A fraction of the newly synthesized delta proteins may directly assemble with the HBV surface proteins at the endoplasmic reticulum, bypassing the nucleus. The result is production of viruslike particles devoid of HDV RNA.

cleus to the cytoplasm may help in the design of novel strategies for antiviral therapy, as this is an essential step in the infectious life cycle of the virus.

MATERIALS AND METHODS

Immunocytochemistry of paraffin-embedded liver sections

Paraffin-embedded liver sections were briefly deparaffinized with xylene and rehydrated with ethanol. Tissue sections were treated with 3% H_2O_2 to inhibit endogenous peroxidase activity, and incubated with Antigen unmasking solution (Vector Laboratories, Burlingame, California) in a microwave for 15 min at 750 W, followed by 5 min at 900 W. After cooling in water, the sections were rinsed with PBS containing 0.05% Tween 20, and treated with avidin and biotin blocking solutions (Vector). For immunocytochemistry, the Histostain DS Double Staining Kit (Zymed, San Francisco, California) was used. All detection procedures were done according to the manufacturer's instructions. This kit employs an affinity-purified bio-

tinylated secondary antibody in conjunction with streptavidin-peroxidase and streptavidin-alkaline phosphatase. Two distinct substrate/chromogen/enzyme systems are used: BCIP/NBT/alkaline phosphatase, which produces a dark purple stain, and hydrogen peroxide/AEC/peroxidase, which produces an intense red. The primary antibodies (rabbit serum B3 and mouse monoclonal antibody NCL-HBsAg-2) were diluted 1:100 and 1:30, respectively, and incubated for 20 min at room temperature.

Cell culture and transfection

Huh7 and NIH 3T3 cells were cultured as monolayers in RPMI 1640 and Dulbecco's Modified Eagle's medium, respectively. Both media were supplemented with 10% fetal calf serum (Gibco BRL) and 1% L-glutamine. Huh7-D12 cells were cultured in the presence of 200 μ g/mL geneticin (G418, Sigma). All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 . One day after being plated onto glass coverslips, Huh7 cells were transfected using FuGene 6 (Roche). The cells were analyzed at 24 h posttransfection.

Immunofluorescence and in situ hybridization

Cells on coverslips were rinsed once in phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After rinsing in PBS containing 0.05% Tween 20 (PBS-Tw), the cells were incubated with primary antibodies for 1 h at room temperature, washed with PBS-Tw, and then incubated with the appropriate secondary antibodies for 30 min at room temperature. After washing, the coverslips were incubated with DAPI (500 ng/mL in PBS for 5 min), mounted in Vecta Shield (Vector Laboratories, UK) and sealed with nail polish.

In situ hybridization was performed using pSVL(D3) as a probe. Plasmid pSVL(D3) contains a trimer of full-length genomic HDV cDNA cloned in pSVL, a eukaryotic expression vector from Pharmacia Biotech. The plasmid was labeled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Germany) by nick translation. The cells were rinsed once in PBS, fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 and 0.5% Saponin in PBS for 10 min at room temperature. Hybridization was performed as previously described (Cunha et al., 1998).

Fluorescent labeled samples were analyzed using the laser scanning microscope Zeiss LSM 510 equipped with an Argon Ion laser (488 nm) to excite FITC fluorescence and a helium-neon laser (543 nm) to excite Texas Red fluorescence.

Heterokaryon assays

Huh7-D12 and Huh7 cells were grown to subconfluency on glass coverslips for 1 day. NIH 3T3 cells were then added in the presence or absence of emetine at 40 μ g/mL, and left to attach for 3 h. To induce cell fusion, the coverslips were first rinsed in PBS and placed on a drop of polyethylene-glycol (PEG 1500; Boehringer Mannheim) for 2 min, washed twice in PBS, and further incubated in fresh culture medium either in the presence or absence of emetine.

Temperature shift assay

Approximately 24 h after transfection, Huh 7 cells were incubated in fresh medium containing 40 μ g/mL emetine for 3 h at 37 °C. The coverslips were then transferred to fresh medium containing 40 μ g/mL emetine and 20 mM HEPES buffer, and placed on ice for 3 h.

SDS-PAGE and immunoblotting

Total cellular proteins were extracted and separated by SDS-PAGE on 12% polyacrylamide minigels (BioRad Laboratories, Richmond, California), electroblotted onto nitrocellulose and incubated with antibodies as previously described (Cunha et al., 1998).

Antibodies

The antibodies used in this study included rabbit serum B3 directed against the small and large form of the delta antigen

(Saldanha et al., 1990), monoclonal antibody NCI-HBsAg-2 (NovoCastra Laboratories, UK), which reacts with HBV surface antigen, and monoclonal antibody 4F4 directed against hnRNP C protein (Choi & Dreyfuss, 1984). Secondary antibodies conjugated to FITC or Texas Red were purchased from Jackson ImmunoResearch Laboratories, USA.

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REFERENCES

- Bell P, Brazas R, Ganem D, Maul GG. 2000. Hepatitis delta virus replication generates complexes of large hepatitis delta antigen and antigenomic RNA that affiliate with and alter nuclear domain 10. *J Virol* 11:5329–5336.
- Braun IC, Rohrbach E, Schmitt C, Izaurralde E. 1999. TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus. *EMBO J* 7:1953–1965.
- Casey JL, Gerin JL. 1995. Hepatitis D virus RNA editing: Specific modification of adenosine in the antigenomic RNA. *J Virol* 12:7593–7600.
- Chang J, Sigal LJ, Lerro A, Taylor J. 2001. Replication of the human hepatitis delta virus genome is initiated in mouse hepatocytes following intravenous injection of naked DNA or RNA sequences. *J Virol* 7:3469–3473.
- Chao M, Hsieh SY, Taylor J. 1990. Role of two forms of hepatitis delta virus antigen: Evidence for a mechanism of self-limiting genome replication. *J Virol* 10:5066–5069.
- Choi YD, Dreyfuss G. 1984. Monoclonal antibody characterization of the C proteins of heterogeneous nuclear ribonucleoprotein complexes in vertebrate cells. *J Cell Biol* 6:1997–2004.
- Chou HC, Hsieh TY, Sheu GT, Lai MM. 1998. Hepatitis delta antigen mediates the nuclear import of hepatitis delta virus RNA. *J Virol* 5:3684–3690.
- Cunha C, Monjardino J, Cheng D, Krause S, Carmo-Fonseca M. 1998. Localization of hepatitis delta virus RNA in the nucleus of human cells. *RNA* 6:680–693.
- David E, Rahier J, Pucci A, Camby P, Scevens M, Salizzoni M, Otte JB, Galmarini D, Marinucci G, Ottobrelli A, et al. 1993. Recurrence of hepatitis D (delta) in liver transplants: Histopathological aspects. *Gastroenterology* 4:1122–1128.
- Eble BE, Lingappa VR, Ganem D. 1986. Hepatitis B surface antigen: An unusual secreted protein initially synthesized as a transmembrane polypeptide. *Mol Cell Biol* 5:1454–1463.
- Ernst RK, Bray M, Rekosh D, Hammarskjöld ML. 1997. Secondary structure and mutational analysis of the Mason-Pfizer monkey virus RNA constitutive transport element. *RNA* 2:210–222.
- Filipovska J, Konarska MM. 2000. Specific HDV RNA-templated transcription by pol II in vitro. *RNA* 1:41–54.
- Ganem D. 1991. Assembly of hepatitis delta virus virions and subviral particles. *Curr Top Microbiol Immunol* 168:61–83.
- Gorlich D, Kutay U. 1999. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 15:607–660.
- Govindarajan S, Chin KP, Redeker AG, Peters RL. 1984. Fulminant

- B viral hepatitis: Role of delta agent. *Gastroenterology* 6:1417–1420.
- Izaurrealde E, Adam S. 1998. Transport of macromolecules between the nucleus and the cytoplasm. *RNA* 4:351–364.
- Kuo MY, Chao M, Taylor J. 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: Role of delta antigen. *J Virol* 5:1945–1950.
- Lazinski DW, Taylor JM. 1994. Expression of hepatitis delta virus RNA deletions: *cis* and *trans* requirements for self-cleavage, ligation, and RNA packaging. *J Virol* 5:2879–2888.
- Lee CH, Chang SC, Wu CH, Chang MF. 2001. A novel chromosome region maintenance 1-independent nuclear export signal of the large form of hepatitis delta antigen that is required for the viral assembly. *J Biol Chem* 11:8142–8148.
- Lee CZ, Chen PJ, Chen DS. 1995. Large hepatitis delta antigen in packaging and replication inhibition: Role of the carboxyl-terminal 19 amino acids and amino-terminal sequences. *J Virol* 9:5332–5336.
- Michael WM, Choi M, Dreyfuss G. 1995. A nuclear export signal in hnRNP A1: A signal-mediated, temperature-dependent nuclear protein export pathway. *Cell* 3:415–422.
- Monjardino J, Lai NMC. 1993. Viral hepatitis. In: Zuckerman T, ed. *Structure and molecular virology*. Churchill Livingstone. pp 329–340.
- Pinol-Roma S, Dreyfuss G. 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* 6362:730–732.
- Ryu WS, Bayer M, Taylor J. 1992. Assembly of hepatitis delta virus particles. *J Virol* 4:2310–2315.
- Saldanha J, Homer E, Goldin R, Thomas HC, Monjardino J. 1990. Cloning and expression of an immunodominant region of the hepatitis delta antigen. *J Gen Virol* 71:471–475.
- Smedile A, Rizzetto M, Gerin JL. 1994. Advances in hepatitis D virus biology and disease. In: Boyer JL, Okner RK, eds. *Progress in liver disease*. Vol 12. Philadelphia: WB Saunders. pp 157–175.
- Sureau C, Moriarty AM, Thornton GB, Lanford RE. 1992. Production of infectious hepatitis delta virus in vitro and neutralization with antibodies directed against hepatitis B virus pre-S antigens. *J Virol* 2:1241–1245.
- Taylor JM. 1992. The structure and replication of hepatitis delta virus. *Annu Rev Microbiol* 46:253–276.